Investigation of the Toxic & Teratogenic effects of GRAS Substances to the Developing Chicken Embryo **(Caffine)** 1/31/74

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SUBJECT: Investigation of the Toxic and Teratogenic Effects of GRAS Substances to the Developing Chicken Embryo

Attached is the report of the investigation of CAFFEINE in the developing chicken embryo.

Investigations of the Toxic and Teratogenic Effects of GRAS Substances to the Developing Chicken Embryo:

CAFFEINE

PROTOCOL:

Caffeine (1) was tested for toxic and teratogenic effects to the developing chicken embryo under four sets of conditions. It was administered, with 5% water solution of glucose as the diluent, by two routes and at two stages of embryonic development; via the air cell at pre-incubation (0 hours) and at 96 hours of incubation, and via the yolk at 0 hours and at 96 hours using techniques that have been described previously (2, 3). For the purpose of determining the possible effect of the glucose solution as the solvent, double distilled water was substituted for the 5% water solution of glucose in a test in which a smaller number of eggs were treated via the air cell.

Groups of ten or more eggs were treated under these four conditions at several dose levels until a suitable total number of eggs per level was reached for all levels allowing some to hatch. Groups of adequate size were treated solely with the solvent at corresponding volumes. Untreated controls were also included in each experiment.

After treatment, all the eggs were candled daily and the non-viable embryos were removed. Surviving embryos were allowed to hatch. Hatched chicks and non-viable embryos were examined grossly for abnormalities (internally and externally) as well as for toxic responses such as edema and hemorrhage. Along with these, histological examinations of major organs (liver, heart, kidney, lung, brain, intestine, gonad, and some endocrine organs) were carried out by taking samples from a representative number of animals from each experimental group.

RESULTS:

The results obtained are presented in Tables 1 through 4 for each of the four conditions of the test.

Columns 1 and 2 give the dose administered in milligrams per egg and milligrams per kilogram egg weight, respectively. (The milligrams per kilogram figure is based on an average egg weight of fifty grams.)

Column 3 is the total number of eggs treated. This number has not been corrected for the sterile eggs or the eggs discarded due to accidents, thus providing a slightly higher mortality rate and a lower abnormality rate than was the actual case.

Column 4 is the percent mortality, i.e., the total number of non-viable eggs divided by the total number of treated eggs.

Column 5 is the total number of abnormal birds expressed as a percentage of the total number of eggs treated. This includes all the abnormalities observed and also the toxic responses such as edema, hemorrhage, hypopigmentation of the down and other disorders such as feather abnormalities, significant growth retardation, cachexia, and neural disorders including ataxia.

Column 6 is the total number of birds having a structural abnormality of the head, viscera, limbs, or body skeleton expressed as a percentage of the total number of eggs treated. Toxic responses and disorders such as those noted for column 5 are not included.

The comparable data for the solvent treated eggs and the pierced and drilled controls as well as the untreated controls are included in columns 3 through 6.

The mortality data in column 4 have been examined for a linear relationship between the probit percent mortality versus the logarithm of the dose according to the procedures of Finney (4). The results obtained are indicated at the bottom of each table.

The data in columns 4, 5 and 6 have been analyzed using the Chi Square test for significant differences from the solvent background. Each dose level is compared to the solvent value and levels that show differences at the 5% level or lower are indicated by an asterisk in the table.

DISCUSSION:

Caffeine was not found to be embryotoxic when administered to the embryos under all of the conditions of the test. The toxicity was significantly (P=0.05) greater than it was for the solvent-treated eggs only at the dose level of 5.0 mg/egg (the highest dose level tested), via the air cell both at 0 hours and 96 hours. Probit analysis resulted in an LC_{50} of 2.689 mg/egg (air cell at 0 hours, Table 1) and an LC_{50} of 11.146 mg/egg (air cell at 96 hours, Table 2). Yolk treatment, however, resulted in a negative slope in both the 0 hours and the 96 hours administration (Tables 3 and 4).

Abnormal birds were seen under all of the conditions of the test, although the incidence of birds having a structural abnormality of the head, limbs, viscera, or skeleton was not significantly different from that of the solvent background (P=0.05). Of the 126 untreated control embryos, only one was abnormal, with curled toes which is a very frequently seen minor anomaly.

AIR CELL AT 0 HOURS: At 5.0 mg/egg there was one bird with celosomia. At 2.5 mg/egg two birds were abnormal, one with hip contracture and the other with curled toes. At 1.0 mg/egg one had curled toes. The solvent-treated eggs had only one abnormality, a bird with hip contracture.

AIR CELL AT 96 HOURS: At each of 5.0 mg/egg and 0.5 mg/egg levels there were two abnormal birds, one with celosomia and the other with hip contracture. The solvent-treated group had one bird with curled toes.

YOLK AT 0 HOURS: The 2.5 mg/egg level was the only level that exhibited an abnormality, one bird with hip contracture. There was no malformed bird from the solvent-treated eggs.

YOLK AT 96 HOURS: The 1.0 mg/egg level was the only level that has shown an abnormality of one bird with hip contracture. No abnormality was found with the solvent-treated birds.

The results from the air cell experiment in which double distilled water replaced the 5% water solution of glucose as the solvent are shown in Tables 5 and 6. As in the groups that used the glucose solution (Tables 1 and 2), the toxicity was significantly different from that of the solvent-treated controls only at or above the dose level of 5.0 mg/egg. For teratogenicity, only one bird with hip contracture was found to be abnormal at 1.0 mg/egg at 0 hours; at 96 hours, four abnormal birds were found only at 1.0 mg/egg level, two birds with hip contracture, one with celosomia and the fourth one with curled toes.

When these results from the experiments using the two different solvents for caffeine are compared, it becomes clear that there is virtually no difference in the effect between these two solvents on either the toxicity of the caffeine or the incidence and variety of the abnormalities found.

Microscopical examination of the paraffin embedded and H&E stained sections revealed no consistent histological changes in any of the organs observed. Although occasional hemorrhage, vacuolization, or fatty infiltration in the liver were seen, neither of these changes correlated with the administered dose nor the varieties of the external abnormalities.

Judging from all of these test results, it is concluded that caffeine is neither toxic nor teratogenic to the chicken embryo below the dose of 5.0 mg per egg under the test conditions used. Most of the abnormalities found in this test were also observed in the solvent-treated or untreated controls.

1. Caffeine (anhydrous), FDA 71-44

2. McLaughlin, J., Jr., Marliac, J.-P., Verrett, M.J., Mutchler, M.K. and Fitzhugh, O.G. Toxicol. Appl. Pharmacol. 5:760-770, 1963

3. Verrett, M.J., Marliac, J.-P. and McLaughlin, J., Jr. JAOAC 47: 1002-1006, 1964

4. Finney, D.J. Probit Analysis, 2nd ed., Cambridge Press, Cambridge, Appendix I, 1964

Table 1

Caffeine

Air Cell at 0 Hours

Dose		Number	Percent	Percent Abnormal	
mg/egg	mg/kg	of eggs	Mortality	Total	Structural
5.0	100	90	93.33*	1.11	1.11
2.5	50	91	74.72	2.19	2.19
1.0	20	93	75.26	1.07	1.07
0.5	10	91	78.02	0	0
5% Glucose Water Solution		90	66.66	1.11	1.11
Controls		126	61.90	0.79	0.79

LC_{30} 0.936 mg/egg (18.724 mg/kg)

LC_{50} 2.689 mg/egg (53.792 mg/kg)

LC_{90} 35.463 mg/egg (709.266 mg/kg)

*Significantly different from solvent $P \leq 0.05$

Table 2

Caffeine

Air Cell at 96 Hours

Dose		Number	Percent	Percent Abnormal	
mg/egg	mg/kg	of eggs	Mortality	Total	Structural
5.0	100	71	76.05*	2.81	2.81
2.5	50	72	59.72	0	0
1.0	20	70	52.85	0	0
0.5	10	71	73.23	2.81	2.81
5% Glucose Water Solution		68	52.94	1.47	1.47
Controls		126	61.90	0.79	0.79

LC_{30} 0.900 mg/egg (18.017 mg/kg)

LC_{50} 11.146 mg/egg (222.928 mg/kg)

*Significantly different from solvent $P \leq 0.05$

Table 3

Caffeine

Yolk at 0 Hours

Dose		Number	Percent	Percent Abnormal	
mg/egg	mg/kg	of eggs	Mortality	Total	Structural
5.0	100	31	100.00	0	0
2.5	50	30	96.66	3.33	3.33
2.0	40	68	97.05	0	0
1.5	30	70	97.14	0	0
1.0	20	102	95.09	0	0
0.5	10	101	94.05	0	0
5% Glucose Water Solution		100	96.00	0	0
Controls		126	61.90	0.79	0.79

Slope is negative

*Significantly different from solvent $P \leq 0.05$

Table 4

Caffeine

Yolk at 96 Hours

Dose		Number	Percent	Percent Abnormal	
mg/egg	mg/kg	of eggs	Mortality	Total	Structural
2.0	40	85	81.17	0	0
1.5	30	95	80.00	0	0
1.0	20	97	73.19	1.03	1.03
0.5	10	94	80.85	0	0
5% Glucose Water Solution		91	78.02	0	0
Controls		126	61.90	0.79	0.79

Slope is negative

*Significantly different from solvent $P \leq 0.05$

Table 5

Caffeine

Air Cell at 0 Hours

Dose		Number	Percent	Percent Abnormal	
mg/egg	mg/kg	of eggs	Mortality	Total	Structural
10	200	20	100.00	0	0
5	100	39	100.00*	0	0
2.5	50	13	61.53	0	0
1.0	20	36	94.44	2.77	2.77
0.5	10	14	42.85	0	0
Water		36	80.55	0	0
Controls		126	61.90	0.79	0.79

LC_{30} 2.620 mg/egg (52.416 mg/kg)

LC_{50} 2.785 mg/egg (55.703 mg/kg)

LC_{90} 3.231 mg/egg (64.631 mg/kg)

*Significantly different from solvent $P \leq 0.05$

Table 6

Caffeine

Air Cell at 96 Hours

Dose		Number	Percent	Percent Abnormal	
mg/egg	mg/kg	of eggs	Mortality	Total	Structural
10	200	24	87.50*	0	0
5	100	33	66.66	0	0
2.5	50	14	50.00	0	0
1.0	20	28	46.42	14.28	14.28
0.5	10	11	9.09	0	0
Water		17	47.05	0	0
Control		126	61.90	0.79	0.79

Slope is negative

*Significantly different from solvent $P \leq 0.05$